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Targeted cytosolic delivery of hydrogel nanoparticles into HepG2 cells through engineered Sendai viral envelopes

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Abstract Hydrogel nanoparticles of cross-linked polyvinylpyrrolidone (PVP-NP) (35–50 nm in diameter) containing fluoresceinated dextran (FITC-Dx) were encapsulated in reconstituted Sendai viral envelopes containing only the fusion (F) protein (F-virosomes[1]). Incubation of these loaded F-virosomes with human hepatoblastoma cells (HepG2) in culture resulted in membrane-fusion-mediated delivery of NPs to the cell cytoplasm, as inferred from the ability of cells to internalize FITC-Dx loaded PVP-NP (PVP^f-NP) in the presence of azide (an inhibitor of the endocytotic process). Introduction of PVP^f-NP into the HepG2 cells was assured by selective accumulation of FITC fluorescence in the cytosolic compartment. The structural integrity of the internalized PVP^f-NP was also confirmed by fluorescence microscopy and ultracentrifugation analysis. The potential usefulness of PVP-NP-mediated cytosolic release of water soluble drugs both in vitro and in vivo has been established for the first time.

Key words: Hydrogel nanoparticle; Sendai virus; Virosome; Drug delivery; Targeted cytosolic delivery; Controlled release

1. Introduction

Despite improvements in viral and non-viral vector systems, a major hurdle in the delivery of drugs and other macromolecules into the desired cell types is crossing the permeability barrier imposed by the plasma membrane followed by the controlled release inside the cytoplasm [1]. On the other hand, it is known that controlled release of drugs can be significantly improved by using nanoparticulate carriers [1]. Among such carriers, ultralow-sized hydrogel nanoparticles [2] made up of polyvinylpyrrolidone (PVP-NP) have been selected as the polymer component and are highly biocompatible [3]. Besides significant progress made in the field of biomedical applications of nanotechnology, different factors (size, surface, cell-specific uptake of NPs and endosomal escape) associated with NP targeting of drugs could be pivotal for a successful outcome [4]. While size, surface and cell-specific uptake of NPs have been resolved to some extent [1], existing technology fails to deliver drug-loaded NPs directly inside the cytosol of desired cell types evading endosomal pathway for its expected optimum action [4]. In the past few years, engineered Sendai viral envelopes (F-virosomes) are known to specifically bind and fuse efficiently with the plasma membrane of hepatocytes both in vitro and in vivo and served as excellent carriers for fusion-mediated microinjection of various biologically active molecules into their cytosolic compartments [5–10].

In the present study, PVP-NP of defined size (approximately 50 nm in diameter) loaded with fluorescein isothiocyanate dextran (FITC-Dx; model drug of choice) have been entrapped within F-virosomes. We demonstrate that these loaded F-virosomes (delivery vehicle inside a delivery vehicle) fuse with HepG2 cell membranes and microinject the encapsulated FITC-Dx-loaded polyvinylpyrrolidone NP (PVP^f-NP) into the cell cytoplasm. The delivery of PVP^f-NPs from virosomes to the cytosol of the target cells is reported for the first time in a systematic and quantitative fashion. The efficacy of this unique hybrid delivery system with respect to controlled release of drugs inside the cytosolic compartment is discussed.

2. Materials and methods

2.1. Preparation of PVP^f-NP

PVP^f-NP were made following our previous communication [11]. In brief, 40 ml of 0.03 M sodium bis-2-ethylhexylsulfosuccinate (AOT) solution in hexane was taken and to it 280 μl of freshly distilled vinyl pyrrolidone, 50 μl of *N*,*N*′-methylene bisacrylamide (0.049 g/ml), 20 μl *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and 20 μl of ammonium persulfate (saturated solution) and 50 μl (160 mg/ml in water) of FITC-Dx (Sigma, St. Louis, MO, USA; mol. wt. 19.3 kDa) were added. Polymerization was carried out at 37°C for 8 h with continuous stirring. After the completion of the reaction the excess solvent (hexane) was evaporated in a rotatory evaporator and the dry mass was resuspended in 10 ml of water by sonication. Then, 1 ml of 30% $CaCl_2$ solution was added dropwise with continuous stirring to precipitate the AOT as calcium salt of bis (2-ethyl-hexyl) sulfosuccinate ($Ca(DEHSS)_2$) followed by dialysis against water. $Ca(DEHSS)_2$ was separated by centrifugation. After separating the unentrapped FITC-

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[1] Process for Producing a Targeted Gene (1997) US Patent 5, 683, 866).

Abbreviations: ASGP-R, asialoglycoprotein receptor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetate (disodium salt); F, fusion protein; FCS, fetal calf serum; FITC-Dx, fluorescein isothiocyanate dextran (mol wt. 19.3 kDa); PL, polylysine; PVP^f-NP, FITC-Dx-loaded polyvinylpyrrolidone nanoparticle; TBS, Tris-buffered saline

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Dx by gel filtration, the supernatant containing PVP^f-NP was lyophilized to dry powder for subsequent use. The amount of FITC-Dx entrapped inside PVP-NP was calculated (200 μg of FITC-Dx/mg of PVP^f-NP) and their average diameter was determined by electron microscopy [2].

2.2. Preparation of PVP^f-NP loaded F-virosomes

Reconstituted Sendai viral envelopes containing F-protein (F-virosome) were prepared as described earlier [5]. The Triton X-100 (TX-100)-solubilized fraction of virus was mixed with PVP^f-NP (500 μg of PVP^f-NP/mg of viral protein) and reconstituted by stepwise removal of detergent using SM2 biobeads. The unentrapped PVP^f-NP adsorbed on the surface of virosomal membrane was removed by repeated washing with cold 0.01 M Tris-buffered saline (TBS), pH 7.4. Furthermore, to remove the residual amount of aggregated form of PVP^f-NP and FITC-Dx adsorbed on the virosomal surface, the loaded virosomes were subjected to sucrose density gradient ultracentrifugation following the protocol of Paternoster et al. [12]. The amount of entrapped PVP^f-NP was determined by fluorescence measurement at 490/520 nm (excitation/emission). FITC-Dx-loaded F-virosomes were made following our published procedure for entrapping water soluble molecules [5,7]. Each preparation of such loaded virosomes was tested for both structure and function following our published protocol [6].

2.3. Cells

HepG2 (human hepatoblastoma cells), NIH3T3 (mouse fibroblast) and CHO (Chinese hamster ovary) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), sodium pyruvate (110 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) under 5% CO_2. All cell culture reagents were procured from Invitrogen, USA.

2.4. Fusion-mediated delivery of PVP^f-NP and FITC-Dx into HepG2 cells

2.4.1. Spectrofluorimetric measurements. Single cell suspensions of HepG2 cells were made by lifting the cells from monolayer with ethylenediaminetetraacetate (EDTA) [7]. The cells were finally suspended in DMEM without serum to obtain 1.0×10^6 cells/ml. Loaded F-virosomes were incubated with cells with varying doses and time at 37°C. The virosome–cell mixtures were washed with cold Dulbecco's phosphate-buffered saline (DPBS) containing 2 mM Ca^{2+} and 2 mM Mg^{2+} thrice and finally incubated at 4°C for 10 min in DPBS containing 5 mM EDTA to remove (EDTA-stripping) the cell surface-bound (unfused) virosomes as standardized earlier [7]. The cell pellets after EDTA-stripping were solubilized in TBS containing 0.3% TX-100 and fluorescence was measured with a spectrofluorometer (model RF 540, Shimadzu Corp., Kyoto, Japan) at 490/520 nm (excitation/emission) wavelength. Heat-treated F-virosomes (incubated at 56°C for 20 min), F-virosomes incubated with NIH3T3 and CHO cells and F-virosomes pre-incubated with mouse anti-F glycoprotein antibody raised in our laboratory [13] were taken as appropriate controls. Fluorescence value of virosome–cell complex without EDTA-stripping was used to calculate the percentage of internalization of PVP^f-NP and/or FITC-Dx by liver cells. Sodium azide is known to be an efficient inhibitor of endocytosis process [14]. Binding and fusion of virosomes and heat-treated virosomes with target cells were studied in the presence of 10 mM azide as described earlier [6,7].

2.4.2. Fluorescence microscopy. Glass coverslips (Erie Scientific, Portsmouth, NH, USA) were coated with polylysine (PL; Sigma, USA) following standard protocol [15]. HepG2 cells were grown in 24-well plates (Becton Dickinson, Cockeysville, MD, USA) on PL-coated coverslips and were incubated with either PVP^f-NP or FITC-Dx-loaded F-virosomes in DMEM without serum. HepG2 cells mixed with heat-treated loaded F-virosomes were used as controls. The medium was replaced after 2 h with DMEM containing 10% FCS and 10 mM sodium azide and was incubated inside a CO_2 incubator for different times. At each time point coverslips were subjected to EDTA-stripping and were mounted on glass slides with 10 μl of DMEM. Fluorescence was detected and images were recorded on Kodak color film using an Eclipse TE 300 (Nikon, Japan) microscope. The light source was a 100 W Hg lamp with appropriate levels of heat and neutral density filters. The fluorescein filters were a standard Nikon set, consisting of 490-nm excitation filter and a 520-nm emission filter. The camera system was from Nikon, Japan.

2.5. Cell fractionation

F-virosomes (PVP^f-NP or FITC-Dx loaded, untreated or heat-treated in each case) were incubated with HepG2 cells in DMEM without serum at 37°C for 2 h. After EDTA-stripping the cells were washed thrice with TBS and were subjected to subcellular fractionation following our published procedure [7]. In brief, the cells were resuspended in the isotonic homogenizing buffer (0.01 M Tris–HCl, pH 7.4, containing 0.25 M sucrose) and then dispersed in a Potter–Elvehjem type homogenizer at 4°C. Fluorescence associated with each fraction was analyzed and expressed as percent of total amount of fluorescence associated with the whole cells. PVP^f-NPs are known to be pelleted at $100\,000\times g$ for 4 h at 4°C without affecting their structural integrity (unpublished observation). Therefore, cytosolic fractions obtained from subcellular fractionation of HepG2 cells were further centrifuged at $100\,000\times g$ for 4 h at 4°C with a view to separate the intact PVP^f-NP from its degraded counterpart.

3. Results

3.1. Characterization of PVP^f-NP loaded F-virosomes

The purity of F-virosome preparations was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol [6] and was found to be free from any contamination by other proteins. Membrane-fusion activity of these loaded virosomes was ascertained by their ability to hemolyze mouse red blood cells (RBCs) in the presence of wheat germ agglutinin [6]. The entrapped PVP^f-NP was retained in intact form inside F-virosomes in the presence of 10% FCS over a period of 48 h in contrast to our earlier observations indicating the ability of serum proteases in degrading free PVP^f-NP [2]. 5–15 μg of PVP^f-NP was encapsulated in 1 mg of F-virosomes whereas 40–60 μg of free FITC-Dx was entrapped per mg of F-virosomes. Electron microscopy of PVP^f-NP (data not shown) preparations and negatively stained loaded virosomal suspension [16] revealed their spherical shape with size varying between 35–50 nm and 100–200 nm in diameter respectively.

3.2. Internalization of PVP^f-NP into HepG2 cells delivered through F-virosomes

Our laboratory has pioneered the observation that F-virosomes can bind and fuse efficiently with HepG2 cells through the interaction of terminal galactose of fusion with the asia-

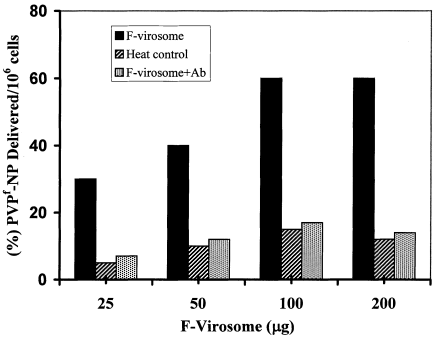

Fig. 1. Dose-dependent internalization of PVP^f-NP into HepG2 cells delivered through F-virosomes. Different amounts (in terms of F protein) of loaded F-virosomes, heat-treated F-virosomes or F-virosomes pre-incubated with anti-F antibody were incubated with HepG2 cells in DMEM at 37°C for 2 h. PVP^f-NP-associated fluorescein with cells was determined after EDTA-stripping as described on Section 2.4. Black bars, F-virosomes with HepG2 cells; hatched bars, heat-treated F-virosomes with HepG2 cells; dotted bars, F-virosomes pre-incubated with anti-F antibody. Each value is mean of three independent determinations.

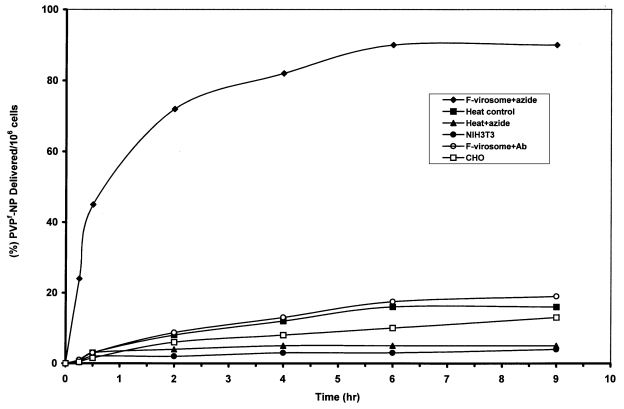

Fig. 2. Kinetics of accumulation of PVP^f-NP into HepG2 cells delivered through F-virosomes. Loaded F-virosomes (100 μg) were incubated with 1.0×10^6 HepG2 cells at 37°C for different times in DMEM. The virosome–cell complexes were kept at 4°C for 10 min in DPBS containing 5 mM EDTA to remove surface bound F-virosomes (unfused). After washing, the cell pellets were solubilized in TBS containing 0.3% TX-100 and fluorescence were measured with a spectrofluorometer. Fluorescence unit without EDTA-stripping was considered as 100%. ♦, F-virosomes with HepG2 cells in the presence of azide; ■, heat-treated F-virosomes incubated with HepG2 cells without azide; ▲, heat-treated F-virosomes with HepG2 cells in the presence of azide; ●, F-virosomes with NIH3T3 cells; ○, F-virosomes pre-incubated with anti-F antibody; □, F-virosomes with CHO cells. The values are means of triplicate observations.

loglycoprotein receptors (ASGP-Rs) [5,7]. We have utilized this system to explore the possibility of delivering PVP^f-NP of defined size to the cytosolic compartment of HepG2 cells. Uptake of PVP^f-NP by cells at 37°C was examined as a function of dose of F-virosomes. Fig. 1 shows a saturation type curve representing cell-associated PVP^f-NP. No increase of PVP^f-NP delivery was noticed beyond 100 μg of F-virosomes and this was employed in all subsequent experiments. Figs. 2 and 3 demonstrate that the initial rate of delivery of PVP^f-NP to HepG2 cells through F-virosomes was twice that of FITC-Dx in the presence of 10 mM sodium azide.

The subtle molecular mechanism associated with higher rate of internalization of NP is yet to be understood. Corresponding heat-treated loaded virosomes in the presence of 10 mM azide revealed no internalization of entrapped PVP^f-NP and FITC-Dx. On the other hand, the heat-treated loaded virosomes were able to bind to HepG2 cells to the same extent as that of untreated virosomes at 4°C (data not shown). Slight internalization (ca. 10%) of both PVP^f-NP and FITC-Dx observed in the case of heat-treated F-virosomes and virosomes pre-incubated with anti-F antibody may be due to the endocytosis of bound non-fusogenic virosomes. Negligible fusion activity (< 2%) was obtained with NIH3T3 and CHO cells as target. Taken together, these results strongly support target-specific, membrane-fusion-mediated cytosolic delivery of PVP^f-NP by F-virosomes to the HepG2 cells.

Table 1
Subcellular Localization of PVP^f-NP inside HepG2 cells

Virosomes	Lysosomal/mitochondrial (%)	Cytosol (%)
Experimental	27	61
Heat-treated	50	21

HepG2 (2.0×10^6) cells were incubated with loaded F-virosomes or heat-treated F-virosomes (200 μg F protein) in DMEM at 37°C for 2 h. The cells were EDTA-stripped and cell fractionation was carried out as described in Section 2. Fluorescence unit without EDTA-stripping was considered as 100%. Each value is the mean of three independent experiments.

Table 2
Integrity of PVP^f-NP in the cytosolic fraction of HepG2 cells

Time (h)	Supernatant (%)	Pellet (%)
0.5	8	90
2	15	80
4	25	73
6	45	52
9	92	5

HepG2 (2.0×10^6) cells were incubated with loaded F-virosomes (200 μg F protein) in DMEM at 37°C over a period of 9 h as described in Fig. 4. The cells were EDTA-stripped and cell fractionation was carried out as described in Section 2. The cytosolic fractions were further subjected to ultracentrifugation at $100\,000\times g$ at 4°C for 4 h to separate the intact PVP^f-NP (fluorescence value in the pellet) from the degraded ones (fluorescence value in the supernatant). Fluorescence unit of the total cytosolic fraction was considered as 100%, while calculating the percent fluorescence values. Each value is the mean of three independent determinations.

3.3. *Subcellular distribution patterns of PVP^f-NP delivered by F-virosomes and heat-treated F-virosomes: cytosolic delivery of PVP^f-NP*

Table 1 shows that about 61% of the total cell-associated fluorescence was recovered from cytosolic fraction using F-virosome as carrier. By contrast, 50% of the fluorescence was recovered in lysosomal/mitochondrial fraction with a concomitant decrease (66%) in the cytosolic fluorescence in the case of heat-treated F-virosomes. These results clearly support the fusion-mediated delivery of PVP^f-NP by F-virosomes to HepG2 cells. Heat-treated F-virosomes being non-fusogenic [5–7] are likely to be taken up by endocytosis leading to their accumulation and subsequent degradation in lysosomes. Additionally, the integrity of the PVP^f-NP inside the cytosol was assessed directly by pelleting the NPs at higher g force fol-

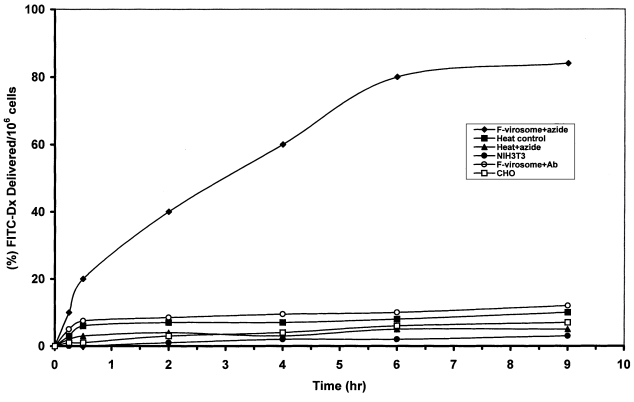

Fig. 3. Kinetics of accumulation of FITC-Dx into HepG2 cells transferred through F-virosomes. Loaded F-virosomes (100 μg) were incubated with 1.0×10^6 HepG2 cells at 37°C for different times in DMEM. For details about the incubation of virosomes and fluorescence measurements, see the legend to Fig. 2. ♦, F-virosomes with HepG2 cells in the presence of azide; ■, heat-treated F-virosomes incubated with HepG2 cells without azide; ▲, heat-treated F-virosomes with HepG2 cells in the presence of azide; ●, F-virosomes with NIH3T3 cells; ○, F-virosomes pre-incubated with anti-F antibody; □, F-virosomes with CHO cells. The values are means of triplicate recordings.

lowed by estimating the associated fluorescence intensity (Table 2).

3.4. Light microscopy of cytosolic delivery of PVP^f-NP in HepG2 cells

To provide direct proof in support of cytosolic localization of PVP^f-NP, we performed additional experiments (Fig. 4). Fig. 4 shows fluorescence microscopy images for the presence of PVP^f-NP inside the HepG2 cells as a result of virosome-mediated delivery in a time-dependent manner (panel A). The release kinetics of FITC-Dx from PVP^f-NP was compared with the delivery of free FITC-Dx through virosomes (diffuse pattern fluorescence, panel B). The intactness of PVP^f-NP was visualized in the form of punctate pattern of FITC fluorescence (arrows) until 6 h post-delivery inside the target cells. Complete release of FITC-Dx from the NPs was observed 9 h post-incubation of HepG2 cells with F-virosomes (cells in panels A and B appeared same) as was confirmed by the absence of any punctate pattern fluorescence. HepG2 cells incubated with heat-treated loaded F-virosomes revealed no detectable fluorescence. The release kinetics of FITC-Dx from NPs appeared to be a slow and well-regulated phenomenon amidst the complex cytosolic milieu of target cells.

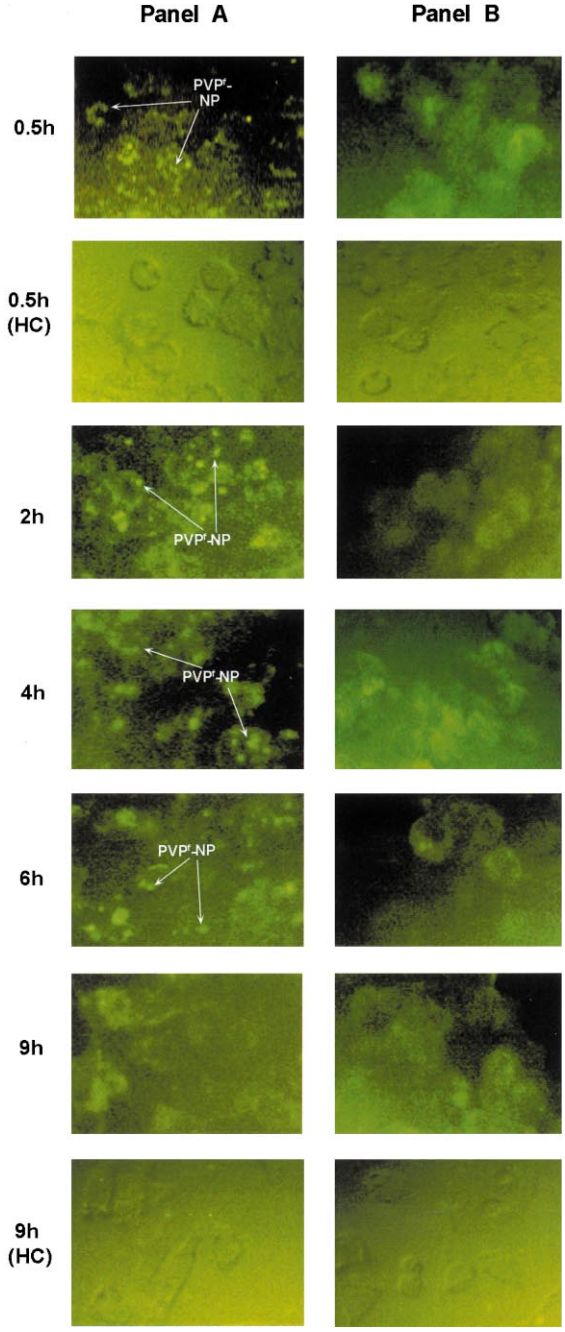

Fig. 4. Controlled release of FITC-Dx from PVP^f-NP into HepG2 cells. HepG2 cells were grown on PL-coated coverslips and were incubated with PVP^f-NP and FITC-Dx-loaded F-virosome separately for different times from 0.5 to 9 h and photographed (magnification, ×40) as mentioned in Section 2. Panel A represents the cells delivered with PVP^f-NP and panel B represents the cells delivered with FITC-Dx through F-virosomes. → denotes the punctate nature fluorescence of PVP^f-NP inside the HepG2 cells; HC stands for heat-treated loaded F-virosomes. This experiment was repeated four times and was highly reproducible.

4. Discussion

In recent years, encapsulation of potent cytotoxic drugs (both hydrophilic and hydrophobic) inside polymeric hydrogel NPs has been proven useful for reducing the toxicity and influencing controlled release of such drugs at the site of action [1,4,17]. However, targeting of such drug-loaded NPs to various cell types both in vitro and in vivo has not yet been precisely achieved [18]. In spite of limited success in conferring cell/tissue specificity on the nanoparticulate surface [1], their in vivo application in targeted drug delivery is severely impeded by the degradation of the polymer at the amide linkages by serum proteases [2]. To circumvent these major obstacles, we have investigated the role of F-virosome in the membrane-fusion-mediated transfer of a model drug-loaded NP to the cytosol of HepG2 cells. The targeted nature of this carrier is apparent from the inability of loaded F-virosomes in transferring PVP^f-NPs to the NIH3T3 and CHO cells (ASGP-R-deficient cell lines). Involvement of plasma membrane level fusion of virosomes with HepG2 cells is confirmed from the failure of azide in inhibiting this transfer process. Heat-treatment of F-virosomes and incubation with specific antibody against F protein are known to completely abolish the fusogenicity of F protein [5–7,13,19]. Very low internalization of PVP^f-NP by heat-treated F-virosomes (and F-virosomes pre-incubated with anti-F antibody) with and without the presence of azide reflects the specificity of this assay. Collectively, these observations corroborate the role of F glycoprotein in specific binding and fusion of a genetically modified Sendai virion and a Sendai mutant (deficient in hemagglutinin–neuraminidase protein) with 1-7-1 cells (NIH3T3 cells stably expressing ASGP-R on the cell surface) and HepG2 cells respectively, as demonstrated earlier [19,20]. The cytosolic delivery of PVP^f-NPs is further ascertained from their subcellular distribution pattern ($>60\%$ in the cytosol) as compared to their heat-treated (ca. 21% in the cytosol) counterparts. Most convincing evidence in support of cytosolic delivery of intact PVP^f-NP is revealed from the punctate pattern of fluorescence in HepG2 cells over a diffuse pattern of free FITC-Dx fluorescence in the presence of azide (a potent inhibitor of endocytosis). Although the size of PVP^f-NPs is below the detection limit of the light microscope, the presence of aggregated NPs produce images (punctate pat-

tern) which are within the range of camera systems. This inference is bolstered by our earlier observations on the interaction of FITC-labeled and octadecylrhodamine B-labeled intact influenza virions with human RBCs [15,21]. Additionally, the delivered PVP^f-NP retained its integrity (punctate pattern) in terms of holding the entrapped FITC-Dx until 6 h. This conclusion was further supported from the ultracentrifugation analysis of cytosolic fractions containing intact PVP^f-NP.

In essence, we have judiciously combined the power of virosomal and nanoparticulate drug delivery systems in designing a novel and efficient hybrid vector to transfer a model drug inside the cytosol of liver cells in culture. As an immediate consequence, the virosome-associated NPs remain protected from degradation by proteases present in the culture media until they are delivered to the cytosol of target cells. On the other hand, once present in the cytosol the NPs mediate slow and controlled release of the entrapped drugs. Therefore, we envisage a potential application of this hybrid vector in vivo in targeting appropriate anti-cancer drugs to liver cells. The NP-mediated slow release of drugs in cytosol is one of the coveted goals in treating liver cancer associated with multidrug resistance [22]. Moreover, technology is available in our laboratory to encapsulate several hydrophobic anti-cancer drugs in nanoparticulate carriers [17,23] that can be in turn delivered to the cytosol of hepatocytes through F-virosomes. Further work is needed to extrapolate this study in delivering drug-loaded NPs to liver cells in whole animal through such virosomal formulations.

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